



DECLARATION

I, Katsuhiro YOSHIDA of 238, Nishikananoi, Showa-machi, Kitakatsushika-gun, Saitama-ken, Japan do hereby declare that I am well acquainted with the Japanese language and English language and the attached English translation of an officially certified copy of Japanese Patent Application No. 111601/1999 is a true and correct translation to the best of my knowledge and belief from the Japanese language into English language.

Declared on this 30th day of June, 2006, in Tokyo, Japan

A handwritten signature in black ink, appearing to read "K. Yoshida".

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[Title of Invention] A novel method for determining a nucleic acid using a nucleic acid probe.

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[Title of Invention] NOVEL METHOD FOR DETERMINING NUCLEIC ACID
USING NUCLEIC ACID PROBE

[CLAIMS]

5 [Claim 1] A novel method for determining a concentration of a target nucleic acid by using a nucleic acid probe labeled with a fluorescent dye, which comprises hybridizing said nucleic acid probe labeled with a fluorescent dye to said target nucleic acid, and measuring a change in fluorescence emission from said 10 fluorescent dye after said hybridization relative to fluorescence emission from said fluorescent dye before said hybridization.

[Claim 2] A novel method for determining a concentration of a target nucleic acid according to claim 1, wherein said 15 fluorescent dye is tetramethyl rhodamine or its derivative, BODIPY FL, BODIPY R6G, BODIPY TMR, or BODIPY TR.

[Claim 3] A novel method for determining a concentration of a target nucleic acid according to claim 1 or 2, wherein said target nucleic acid is a nucleic acid contained in cells derived 20 from a microorganism or animal obtained by single colony isolation or a nucleic acid contained in a homogenate of said cells.

[Claim 4] A novel method for determining a concentration of a target nucleic acid according to claim 1 or 2, wherein said 25 target nucleic acid is a nucleic acid contained in cells of a

complex-cultivation system of microorganisms or symbiotic cultivation system of microorganisms or a nucleic acid contained in a homogenate of said cells.

[Detailed Explanation of Invention]

5 [0001]

[Technical Field]

This invention relates to a method for determining a nucleic acid by using the nucleic acid probe. Specifically, the invention is related to a novel method for determining a nucleic acid, 10 comprising hybridizing said nucleic acid probe labeled with a fluorescent dye to said target nucleic acid, and measuring a change in fluorescence emission from said fluorescent dye after said hybridization relative to fluorescence emission from said fluorescent dye before said hybridization.

15 [0002]

[Prior Art]

A variety of methods are conventionally known to determine a concentration of a nucleic acid by using a nucleic acid probe labeled with a fluorescent dye. These methods include a dot blotting assay: after a target nucleic acid and the nucleic acid probe labeled with a fluorescent dye are hybridized on a membrane, 20 unreacted nucleic probe is washed off.

The intensity of fluorescence only from fluorescent dye molecules, by which the nucleic acid probe hybridized with the 25 target nucleic acid is labeled, is measured.

[0003]

Although this method has been now widely used, at least three days are needed for obtain a final data needs. Thereby, this method has been widely not utilized because the method is 5 too time-spending for determining the amount of a nucleic acid of a specified microorganism in a microorganism-growing system, specifically, in a complex-microorganisms system cultivation, in order to control the growth of the specified microorganism. There is, accordingly, a long-standing desire for the 10 development of a nucleic acid determination method which is usable conveniently in such a system also.

[0004]

[Problem to be solved]

With the foregoing in view, the present invention has as 15 an object thereof the provision of a method for determining a concentration of a target nucleic acid by using a nucleic acid probe labeled with a fluorescent dye, which makes it possible to determine the concentration of the target nucleic acid in a shorter time, and more easily.

20 [0005]

[Means for Solving Problem]

To achieve the above-described object, the present inventors have proceeded with a variety of investigations; as results, the inventors have obtained findings such that, on the 25 hybridization of a nucleic acid probe labeled with the

fluorescent dye to a target nucleic acid, the emission of fluorescence from a fluorescent dye decreases, with this decrease being significant with certain specific dyes.

The present invention has been completed based on the
5 above-described findings.

[0006]

That is, 1) the present invention provides a method for determining a concentration of a target nucleic acid, which comprises hybridizing a nucleic acid probe labeled with a
10 fluorescent dye to the target nucleic acid, and measuring a decrease amount in fluorescence of the fluorescent dye before and after the hybridization.

Further, 2) this invention provides a novel method for determining a concentration of a target nucleic acid according
15 to the above 1), wherein said fluorescent dye is tetramethyl rhodamine or its derivative, BODIPY FL, BODIPY R6G, BODIPY TMR, or BODIPY TR.

Still further, 3) this invention provides a novel method for determining a concentration of a target nucleic acid
20 according to the above 1) or the above 2), wherein the target nucleic acid is a nucleic acid contained in cells derived from a microorganism or animal obtained by single colony isolation or a nucleic acid contained in a homogenate of said cells.

In addition, this invention provides a novel method for
25 determining a concentration of a target nucleic acid according

to the above 1) or 2), wherein said target nucleic acid is a nucleic acid contained in cells of a complex-cultivation system of microorganisms or symbiotic cultivation system of microorganisms or a nucleic acid contained in a homogenate of 5 said cells.

[0007]

[Preferable Embodiment of Invention]

The present invention will next be described in further detail based on certain preferred embodiments.

10 The present invention, in a method for determining a concentration of a target nucleic acid, is characterized with the measurement of a decrease in fluorescence emission from the fluorescent dye, which decrease has been caused on the hybridization of the probe to the target nucleic acid and is 15 a different between before and after it.

[0008]

20 The method for determining a target nucleic acid by using a nucleic acid probe labeled with a fluorescent dye is a method for determining a target nucleic acid which is widely used in general at present; for example, a dot-blotting assay method making use of a membrane filter and the like is mentioned.

[0009]

25 These methods comprise hybridizing the nucleic acid probe labeled with a fluorescent dye to a target nucleic acid, removing, subsequent to the hybridization, unreacted nucleic probe from

a determining system in a way of washing off, and measuring the remaining fluorescence amount. This invention is characterized with the possible determination of the target nucleic acid without such complex procedures

5 [0010]

The term "fluorescent dye" as used herein means fluorescent dyes or the like, which are generally used for the determination or detection of nucleic acids by labeling nucleic acid probes. Illustrative of such fluorescent dyes are fluorescein and derivatives thereof [for example, fluorescein isothiocyanate (FITC) and the like]; BODIPY FL (trade name, product of Molecular Probes, Inc., U.S.A.); EDANS (5-(2'-aminoethyl)amino-1-naphthalene sulfonic acid); rhodamine 6G (R6G) and its derivatives [for example, 10 tetramethylrhodamine (TMR), tetramethylrhodamine isothiocyanate (TMRITC)]; BODIPY R6G (trade name, product of Molecular Probes, Inc., U.S.A.); BODIPY TMR (trade name, product of Molecular Probes, Inc., U.S.A.); Texas red or its derivatives [for example, BODIPY TR (trade name, product of Molecular Probes, Inc., U.S.A.)] and the like. Among these, FITC, EDANS, BODIPY 15 FL, BODIPY R6G, BODIPY TMR, BODIPY TR and the like, are preferred, with BODIPY FL being more preferred.

20 [0011]

The terms a "nucleic acid probe" and "hybridization" have 25 the same meaning as the terms at present used in the molecular

biology and gene engineering. The nucleic acid probe according to the present invention comprises an oligonucleotide. Further, as a "nucleic acid", DNA, RNA, a deoxyribooligonucleotide and ribooligonucleotide are referred to. In addition, the nucleic acid may include a modified RNA such as 2'-o-methyl oligoribonucleotide. The nucleic acid targeted in the present invention may be a mixture of the above nucleic acids, or may be mixed with proteins. Further, the nucleic acid may exist in a cell; no particular limitation is imposed on the cell; the cell may be a procaryotic cell or a eukaryotic cell; various cells may be mixed.

[0012]

The nucleic acid probe may be formed of either a deoxyribooligonucleotide or a ribooligonucleotide. Further, it may be such a modified RNA as 2'-o-methyl oligoribonucleotide. In addition, the probe is formed of 5 to 50 bases, preferably 10 to 25 bases, most preferably 15 to 20 bases. A base number greater than 50 leads to lower permeability through a cell membrane to narrow an applicable range of the present invention. A base number smaller than 5, on the other hand, tends to induce non-specific hybridization and, therefore, results in a large determination error.

[0013]

The oligonucleotide in the nucleic acid probe in the present invention can be produced by a conventional production process

for general oligonucleotides. It can be produced, for example, by a chemical synthesis process or by a microbial process which makes use of a plasmid vector, a phage vector or the like (Tetrahedron Letters, **22**, 1859-1862, 1981; Nucleic Acids Research, **14**, 6227-6245, 1986). Further, it is suitable to use a nucleic acid synthesizer currently available on the market (for example, "ABI 394", manufactured by Perkin-Elmer Corp., U.S.A.).

[0014]

To label the oligonucleotide with the fluorescent dye, desired one of conventionally-known labeling methods can be used (Nature Biotechnology, **14**, 303-308, 1996; Applied and Environmental Microbiology, **63**, 1143-1147, 1997; Nucleic Acids Research, **24**, 4532-4535, 1996). To conjugate a fluorescent dye to the 5'end, a linker or spacer, for example, $-(CH_2)_n-SH$ is first introduced at the 5'end by a method known *per se* in the art. As such a linker- or spacer-introduced derivative is available on the market, a commercial product may be purchased (Midland Certified Reagent Company). In the above-mentioned example, n ranges from 3 to 8 with 6 being preferred. The oligonucleotide can be labeled by reacting an SH- reactive fluorescent dye to the spacer. The thus-synthesized oligonucleotide, which is labeled with the fluorescent dye can be purified by using a reversed phase chromatographic method or the like to provide a nucleic acid probe for use in the present

invention.

[0015]

Further, to conjugate the fluorescent dye to the 3' end of the oligonucleotide, a linker, for example, -(CH₂)_n-NH₂ is introduced onto an OH group on the C atom at the 3'-position of ribose or deoxyribose. As such a linker-introduced derivative is also available on the market like the above-described one, a commercial product may be purchased (Midland Certified Reagent Company). In this case, n ranges from 5 to 8, with 4 to 7 being preferred. The oligonucleotide can be labeled by reacting an NH₂- fluorescent dye to the linker. The thus-synthesized oligonucleotide, which is labeled with the fluorescent dye can be purified by using a reversed phase chromatographic method or the like to provide a nucleic acid probe for use in the present invention. For the introduction of the amino group, it is convenient to use a kit reagent [for example, "Uni-link Aminomodifier" (product of Clontech Laboratories, Inc., U.S.A.), or "FluoReporter Kit F-6082, F-6083, F-6084 or F-10220" (product of Molecular Probes, Inc., U.S.A.)]; 10 in a manner known per se in the art, molecules of the fluorescent dye can then be conjugated to the oligo-ribonucleotide. It is also possible to introduce molecules of the fluorescent dye into strands of the probe nucleic acid (ANALYTICAL BIOCHEMISTRY, 225, 15 20 25 32-38, 1998).

25 [0016]

The use of the above-described nucleic acid probe according to the present invention makes it possible to specifically determine a concentration of a target nucleic acid with ease in a short time. A description will hereinafter be made of the 5 determination method.

In the determination method according to the present invention, the nucleic acid probe of the present invention is firstly added to a measurement system and is caused to hybridize to a target nucleic acid. This hybridization can be effected 10 by a conventionally-known method (*Analytical Biochemistry*, **183**, 231-244, 1989; *Nature Biotechnology*, **14**, 303-308, 1996; *Applied and Environmental Microbiology*, **63**, 1143-1147, 1997). As conditions for hybridization, the salt concentration may range from 0 to 2 molar concentration, preferably from 0.1 to 1.0 molar 15 concentration, and the pH may range from 6 to 8, preferably from 6.5 to 7.5.

[0017]

The reaction temperature may preferably be in a range of the T_m value of the nucleic acid hybrid complex, which is to 20 be formed by hybridization of the nucleic acid probe of the present invention to the specific site of the target nucleic acid, \pm 10°C. This temperature range can prevent non-specific hybridization. A reaction temperature lower than $T_m-10^\circ\text{C}$ allows non-specific hybridization, while a reaction temperature 25 higher than $T_m+10^\circ\text{C}$ allows no hybridization. Incidentally, a

T_m value can be determined in a similar manner as in an experiment which is needed to design the nucleic acid probe for use in the present invention. Described specifically, an oligonucleotide which is to be hybridized with the nucleic acid probe of this 5 invention (and has a complementary base sequence to the nucleic acid probe) is chemically synthesized by the above-described nucleic acid synthesizer or the like, and the T_m value of a nucleic acid hybrid complex between the oligonucleotide and the nucleic acid probe is then measured by a conventional method.

10 [0018]

The reaction time may range from 1 second to 180 minutes, preferably from 5 seconds to 90 minutes. If the reaction time is shorter than 1 second, a substantial portion of the nucleic acid probe according to the present invention remains unreacted 15 in the hybridization. On the other hand, no particular advantage can be brought about even if the reaction time is set excessively long. The reaction time varies considerably depending on the kind of the nucleic acid, namely, the length or base sequence of the nucleic acid.

20 In the present invention, the nucleic acid probe is hybridized to the target nucleic acid as described above. The intensity of fluorescence emitted from the fluorescent dye is measured by a fluorimeter both before and after the hybridization, and a change in fluorescence intensity is then calculated. As 25 the decrease is proportional to the concentration of the target

nucleic acid, the concentration of the target nucleic acid can be determined.

[0019]

The method of the present invention can be applied to nucleic acids contained in cells of microorganisms, plants or animals or those contained in homogenates of the respective cells. The method of the present invention can also be suitably applied to nucleic acids in cells of a cultivation system of microorganisms (e.g., a co-cultivation system of microorganisms 10 or a symbiotic cultivation system of microorganisms), in which various kinds of microorganisms are contained together or a microorganism and other animal- or plant-derived cells are contained together and cannot be isolated from each other, or in a homogenate or the like of the cells of the cultivation system. 15 The term "microorganisms" as used herein means microorganisms in general sense, and no particular limitation is imposed thereon. Examples of such microorganisms can include eukaryotic microorganisms and prokaryotic microorganisms, and also mycoplasmas, virus and rickettsias. The term "a target nucleic acid" as used in connection with such a microorganism system 20 means a nucleic acid with a base sequence specific to cells of a cell strain which is desired to be investigated, for example, as to how it is acting in the microorganism strain. Illustrative examples can include 5S rRNAs, 16S rRNAs and 23S rRNAs of certain 25 specific cell strains and particular sequences of their gene

DNAs.

[0020]

According to the present invention, a nucleic acid probe is added to a co-cultivation system of microorganisms or a symbiotic cultivation system of microorganisms and the amount of 5S rRNA, 16S rRNA or 23S rRNA of a particular cell strain or its gene DNA, thereby making it possible to determine the viable count of the particular strain in the system.

Incidentally, a viable count of a particular cell strain in a co-cultivation system of microorganisms or a symbiotic cultivation system of microorganisms can be determined by adding the nucleic acid probe to a homogenate of the system and then measuring the intensity of fluorescence emission from the fluorescent dye before and after hybridization. It is to be noted that this method also falls within the technical scope of the present invention.

[0021]

The above-described determination method can be carried out as will be described hereinafter. Before the addition of the nucleic acid probe of the present invention, the temperature, salt concentration and pH of the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms are adjusted to meet the conditions described above. It is also preferable to adjust the concentration of the specific cell strain, which is contained in the

co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms, to 10^7 to 10^{12} cells/mL, preferably 10^9 to 10^{10} cells/mL in terms of viable count. These adjustments can be achieved by dilution, centrifugal or like concentration, or the like. A viable count smaller than 10^7 cells/mL results in low fluorescence intensity and greater determination error. A viable count greater than 10^{12} cells/mL, on the other hand, leads to excessively high fluorescence intensity from the co-cultivation system of a microorganism or the symbiotic cultivation system of microorganisms, so that the viable count of the particular microorganism cannot be determined quantitatively. However, this range depends upon the performance of a fluorimeter to be used.

[0022]

The concentration of the nucleic acid probe of the present invention to be added depends upon the viable count of the particular cell strain in the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms and, at a viable count of 10^8 cells/mL, may be in a range of from 0.1 to 10.0 nM, preferably in a range of from 0.5 to 5 nM, more preferably 1.0 nM. A probe concentration lower than 0.1 nM cannot provide any data which accurately reflects the viable count of the particular microorganism. The optimal concentration of the nucleic acid probe according to the present invention, however, cannot be specified in any wholesale manner

because it depends upon the concentration of a target nucleic acid in cells.

[0023]

Upon hybridizing the nucleic acid probe to the 5S rRNA, 5 16S rRNA or 23S rRNA of the particular cell strain or its gene DNA in the present invention, the reaction temperature may be set as described above. Further, the hybridization time may also be set as described above.

The nucleic acid probe according to the present invention 10 is hybridized to the 5S rRNA, 16S rRNA or 23S rRNA of the particular cell strain or its gene DNA under such conditions as described above. Intensities of fluorescence from the fluorescent dye in the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms before and after the 15 hybridization are then measured.

[0024]

The decrease amount of fluorescent emission which is measured in the above way is proportional to an existing amount of a specified strain in a complex microbe system or a symbiotic 20 microbe system. It is based on that the amount of 5S rRNA, 16S rRNA or 23S rRNA in the cell of the specific strain or its gene DNA is proportional to the existing amount of the specific strain in the system.

[0025]

25 In the present invention, no particular limitation is

imposed on components other than the microorganisms in the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms, insofar as the components do not interfere with the hybridization between the nucleic acid 5 probe according to the present invention and the 5S rRNA, 16S rRNA or 23S rRNA of the particular cell strain or its gene DNA and further, do not inhibit the emission of fluorescence from the fluorescent dye or the action of the quencher substance labeled on the oligonucleotide. For example, phosphates such 10 as KH_2PO_4 , K_2HPO_4 , NaH_2PO_4 and Na_2HPO_4 , inorganic nitrogens such as ammonium sulfate, ammonium nitrate and urea, various salts of ions such as magnesium, sodium, potassium and calcium, various salts such as the sulfates, hydrochlorides, carbonates and the like of trace metal ions such as manganese, zinc, iron and cobalt, 15 and vitamins may be contained to adequate extent. If the above-described interference or inhibition is observed, it may be necessary to separate cells of the plural microorganisms from the cultivation system by an operation such as centrifugal separation and then to resuspend them in a buffer or the like.

20 [0026]

Usable examples of the buffer can include various buffers such as phosphate buffer, carbonate buffer, Tris-HCl buffer, Tris-glycine buffer, citrate buffer, and Good's buffer. The buffer should be adjusted to a concentration not inhibiting the 25 hybridization or the emission of fluorescence from the

fluorescent dye. This concentration depends upon the kind of the buffer. The pH of the buffer may range from 4 to 12, with 5 to 9 being preferred.

[0027]

5 [Examples]

The present invention will next be described more specifically based on the following Examples.

Example 1

Preparation of a nucleic acid probe to be hybridized to
10 the nucleic acid base sequence of a nucleic acid ranging from the 335th base to 358th base counted from the 5' end in 16S rRNA of *Escherichia coli*, namely, preparation of a nucleic acid probe having the base sequence of (3')CCGCTCACGCATT(5') was conducted as will be described hereinafter.

15 [0028]

Preparation of nucleic acid probe

An oligonucleotide, which was composed of an oligodeoxyribonucleotide having the base sequence of (3')CCGCTCACGCATT(5') and -(CH₂)₇-NH₂ bonded to the OH group at 20 the 5' position of the oligodeoxyribonucleotide, was purchased from Midland Certified Reagent Company, U.S.A. From Molecular Probes, Inc., "FluoReporter Kit F-6082" (trade name) was also purchased, which contained not only "BODIPY FL" propionic acid succinimidyl ester but also a reagent for conjugating the 25 compound to the amine derivative of the oligonucleotide. The

kit was caused to act on the above-purchased oligonucleotide, whereby a nucleic acid probe labeled with "BODIPY FL" was synthesized for use in this Example.

[0029]

5 Purification of synthesized product: The synthesized product was dried into a dry product. The dry product was dissolved in 0.5 M NaHCO₃/Na₂CO₃ buffer (pH 9.0). The solution was subjected to gel filtration through "NAP-25 Column" (trade name, product of Pharmacia AB, Uppsala, Sweden), whereby 10 unreacted substances were removed. Further, reversed phase HPLC (B gradient: 15 to 65%, 25 minutes) was conducted under the below-described conditions. An eluted main fraction was collected. The collected fraction was lyophilized, whereby a nucleic acid probe was obtained with a yield of 23% as calculated 15 relative to 2 mM of the starting oligonucleotide.

[0030]

The above-described reversed phase chromatography was conducted under the following conditions:

Eluting solvent A: 0.05 N TEAA 5% CH₃CN

20 Eluting solvent B (for gradient elution): 0.05 N TEAA
 40% CH₃CN

Column: "CAPCEL PAK C18" , 6 x 250 mm

Elution rate: 1.0 mL/min

Temperature: 40°C

25 Detection: 254 nm

[0031]

Example 2

Using a 200-mL Erlenmeyer flask which had been sterilized and which contained sterilized nutrient broth (NB) (50 mL; 5 product of Difco; composition: NB, 0.08 g/100 mL), *Escherichia coli* JM109 was cultured overnight at 37°C under shaking. To the culture, an equivalent amount of 99.7% ethanol was then added. A 2-mL aliquot of the ethanol-added culture was centrifuged in a 2.0-mL Eppendorf centrifuge tube, whereby cells were obtained. 10 The cells were washed once with 30 mM phosphate buffer (sodium salt) (100 μL; pH 7.2). The cells were suspended in the phosphate buffer (100 μL) which contained 130 mM NaCl. The suspension was ultrasonicated for 40 minutes under ice cooling (output: 33 W, oscillating frequency: 20 kHz, oscillation method: 15 0.5-second oscillation, followed by a 0.5-second pause), whereby a homogenate was prepared.

[0032]

After the homogenate was centrifuged, the supernatant was collected and was then transferred into a cell of a fluorimeter. 20 The cell with the supernatant placed therein was controlled at 36°C. A solution of the above-described nucleic acid probe, said solution having had been controlled to 36°C beforehand, was added to the supernatant to give a final concentration of 5 nM. While controlling at 36°C, *E. coli* 16S rRNA and the nucleic 25 acid probe were hybridized for 90 minutes. Intensity of

fluorescence emission from the fluorescent dye was then measured by the fluorimeter.

[0033]

As the intensity of fluorescence emission from the 5 fluorescent dye before the hybridization, a value measured by using 30 mM phosphate buffer (sodium salt), which contained 130 mM NaCl, (pH: 7.2) instead of the above-described supernatant was adopted. Intensity of fluorescence emission was measured by changing the ratio of the amount of the nucleic probe to the 10 amount of the supernatant (exciting light: 503 nm; measured fluorescence color: 512 nm). The results are shown in FIG. 7. As is appreciated from FIG. 7, the intensity of fluorescence emission from the fluorescent dye decreased as the ratio of the amount of the supernatant increased. Namely, it is understood 15 that in the present invention, the magnitude of a decrease in fluorescence emission from a fluorescent dye becomes greater in proportion to the amount of a target nucleic acid to which a nucleic acid probe hybridizes.

[0034]

20 Example 3

Preparation of nucleic acid probe: An oligonucleotide, which was to be hybridized to 23S rRNA of *Escherichia coli* JM109, had a base sequence of (5') CCCACATCGTTGTCTGGG(3') and contained -(CH₂)₇-NH₂ bonded to the OH group on the carbon atom 25 at the 3' position of the 5' end nucleotide of the oligonucleotide,

was purchased from Midland Certified Reagent Company, U.S.A. as in Example 8. From Molecular Probes, Inc., "FluoroReporter Kit F-6082" was also purchased as in Example 8, which contained not only "BODIPY FL" propionic acid succinimidyl ester but also 5 a reagent for conjugating the compound to the amine derivative of the oligonucleotide. The kit was caused to act on the oligonucleotide, whereby a nucleic acid probe labeled with "BODIPY FL" was synthesized. The synthesized product so obtained was purified as in Example 8, whereby the nucleic acid 10 probe labeled with "BODIPY FL" was obtained with a yield of 25% as calculated relative to 2 mM of the starting oligonucleotide.

[0035]

Example 4

With *Escherichia coli* JM109 cells obtained in Example 9, 15 cells of *Pseudomonas paucimobilis* (now called "*Sphingomonas paucimobilis*") 421Y (FERM P-5122), said cells having have been obtained using the same culture medium and cultivation conditions as in Example 2, were mixed at the same concentration as *Escherichia coli* JM109 in terms of OD660 value, whereby a 20 complex-cultivation system of the microorganisms was prepared. From the resulting mixed system in which the cell concentration of *Escherichia coli* JM109 was the same as that in Example 2, a homogenate was prepared in the same manner as in Example 2. An experiment was conducted in a similar manner as in Example 25 2 except that the nucleic acid probe prepared in Example 3 was

used, results similar to those obtained in Example 2 were obtained.

[0036]

[Advantageous Effect of Invention]

5 The above method for determining a nucleic acid according to the present invention does not required any procedures of removing unreacted nucleic probe from a measuring system; the method makes it possible to determine a target nucleic acid in a short time and in a simple way. On application of the method
10 to a complex-microbe system and symbiotic microbe system, the existing amount of a specific strain in that system is capable of being determined specifically and in a short time.

[Brief Description of the Drawings]

15 [FIG. 1] The figure is a diagram showing measurement data of fluorescence intensity when the sequence of bases in 16S rRNA of *Escherichia coli*, said bases ranging from the 335th base to the 358th base as counted from the 5' end, was determined using a nucleic acid probe obtained in Example 1.

[Name of Document] Abstract

[Abstract]

[Problem to be Solved] To provide a method for determining a nucleic acid using nucleic acid probe labeled with a fluorescent dye, in which the nucleic acid is capable of being determined in shorter time and in more ease.

[Means for Solving Problem] There is provided with a method for determining a nucleic acid using a nucleic acid probe labeled with a fluorescent dye, in which the method comprise hybridizing the nucleic acid probe to a target nucleic acid, and measuring a decrease in the emission of the dye before and after the hybridization.

[Selected Drawing] None



[Name of Document] Drawings

[Fig. 1]

